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Investigation into the Mechanism of B-13 Cell Death Induced By M8OI

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Abstract

Generally liquid at room temperature, ionic liquids (ILs) are a class of charged compounds with minimal volatility. More and more industrial applications are using methylimidazolium ions (MILs). However, evidence indicates that MILs are the most harmful ILs. The (8C) 1-octyl-3-methylimidazolium IL (M8OI) has been found to be a contaminant in the environment and to have evidence of human exposure in recent investigations. Furthermore, M8OI exposure was greater in PBC patients, and M8OI has been suggested as a potential PBC trigger. But nothing is understood about how harmful it is. The research aimed to find a chemical or compounds that can reduce the toxicity of M8OI by looking at the primary mechanism(s) of M8OI toxicity. Using the MTT assay, B-13 cells, a well-known hepatic progenitor cell line, were tested for alterations in viability after being treated with M8OI and/or the compounds of interest. Using the Seahorse XF analyzer, mitochondrial functions were examined. Using a Western blot, apoptosis and mitophagy were assessed. The toxicity of M8OI was lessened by sodium succinate. M8OI

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appears to reduce mitochondrial oxygen consumption rate (OCR) in two mechanistically distinct ways by interfering with the mitochondrial electron transport chain (ETC). While complex I did not reduce OXPHOS, low M8OI concentrations did inhibit mitochondrial OCR. By preventing complex I's OXPHOS oxidation, higher quantities of M8OI inhibited OC in a manner similar to that of rotenone. While it was discovered that the PINK1-Parkin pathway mediates mitophagy activation, M8OI induced apoptosis through both mitochondrial-dependent and independent mechanisms. According to these findings, M8OI and the mitochondrial ETC directly interact twice before B-13 cells undergo apoptosis.

Key words*: ionic liquids (ILs), B-13 Cell Death, Methylimidazolium ILs (MILs), the (8C) 1-octyl-3-methylimidazolium IL (M8OI)*

1. Introduction

1.1 Primary Biliary Cholangitis (PBC)

Addison and Gull made the original identification of PBC in 1851. According to Purohit and Cappell (2015), it is a chronic, autoimmune, cholestatic liver disease that causes inflammation and destruction to the small interlobular and septal bile ducts (those with a diameter of less than 100m). PBC is ten times more common in women than in men, and it rises even more in females beyond the age of fifty (Leitch et al., 2021). Due to the paucity of understanding on the aetiology of PBC, preventative medicinal treatments are limited (Meyer et al., 2017). Potential outcomes of PBC include worsening symptoms, liver transplantation, and PBC development, depending on how well some treatments, such as ursodeoxycholic acid, work.

1.2 Ionic Liquids (ILs)

Ionic liquids (ILs) are referred to by various names, such as molten salts, fused salts, and slat of organic liquids (Bubalo et al., 2017). They all have the trait of low

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volatility and being liquid at room temperature (Welton, 2018). The most often utilized IL cations are imidazolium, ammonium, phosphonium, sulfonium, and pyridinium (Figure 2) (Young et al., 2020). According to Oskarsson and Wright (2019), the ILs market is expected to reach a size of over 50 kilo tonnes and expand at a pace of more than 22% annually. Additionally, since 2015, ILs have been included in more than 3,000 publications annually. As of 2020, the global market for ILs was valued at over $1.40 billion, and estimates indicate that figure will rise to $4.5 billion by 2027 (Wei, et al., 2021).

1.3 Methylimidazolium ILs (MILs)

MILs are made up of a range of different ions and a cationic methylimidazolium moiety with an alkyl chain that lengthens by two carbons (Young et al., 2020). The EU uses between 60 and 70 MILs (Young et al., 2020). According to Young et al. (2020) and Abdelghany et al. (2020), these MILs are probably suggested as solvents and utilized in high concentrations for dissolutions, separations, and catalysis alongside 1-ethyl-3 methylimidazolium acetate. But compared to ILs with pyridinium or morpholinium rings, those with imidazolium heterocyclic rings exhibit higher toxicity (Bubalo et al., 2017).

Studies also confirm that MILs are the most toxic ILs due to the imidazolium ring's hydrogen bonding, which is thought to increase their toxicity (Zhao et al., 2014). The exclusion of MILs from the lists utilized for environmental assessments and biomonitoring (Leitch et al., 2021) is therefore perplexing. Furthermore, a liver progenitor cell line is toxic to 1-octyl-3-methylimidazolium (M8OI) (Figure 2A), which has been found near a land fill/waste site (Probert et al., 2018).

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1.4 M8OI

M8OI is not included in any US home items, EU cosmetics, or the UK Health and Safety Executive COPR Database. In spite of this, the European Chemicals Agency (ECHA) has registered 60 methylimidazolium ILs; however, due to their toxicity dossiers, listing is not mandatory. Five pre-registered M8OI salts are included in the ECHA database; however, due to the current lack of information regarding their toxicity, their production level concentration needs to be less than 100 tonnes annually (Leitch et al., 2020A). Leitch et al. (2021) demonstrated that M8OI is present in the general population, despite their findings being based on a small sample size of 20 PBC patients.

1.5 M8OI and PBC

According to Purohit and Cappell (2015), PBC is thought to be caused by a lack of tolerance to the lipoic acid-conjugated sections of the pyruvate dehydrogenase complex enzyme subunit 2 (PDC-E2). It was shown that the hepatic metabolite of M8OI that contains carboxylates is structurally comparable to lipoic acid. Furthermore, employing recombinant enzymes from the exogenous lipoylation route, the carboxylate-metabolite was successfully integrated into the PDC-E2 in vitro through enzymatic means. For the first time, these results showed that M8OI might be taken into account as a possible xenobiotic environmental trigger for PBC (Probert et al., 2018).

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2. Aims and Hypothesis

2.1 Aims

Looking into the primary mechanism(s) underlying M8OI toxicity and determining the compound(s) that can reduce M8OI toxicity. It is divided into these four sub objectives:

1. To ascertain how M8OI affects specific proteins that either triggers or control mitophagy and apoptosis, respectively.
2. To see if menadione, menadiol, succinate, or apigenin could lessen M8OI's toxicity in B-13 cells.
3. To find out whether M8OI inhibits oxidative phosphorylation by directly interacting with ETC and, if so, which mitochondrial ETC complex (es) M8OI targets.
4. To ascertain whether M8OI obstructs the transport of electrons from CI to CIII.

2.2 Hypothesis

- M8OI induce apoptosis in B-13 cell via mitochondrial pathway.
- M8OI induce mitophagy in B-13 cell via PINK/Parkin signalling pathway.
- M8OI inhibit oxidative phosphorylation, by targeting the mitochondrial ETC and inhibiting
- NADH:CoQ oxidoreductase enzyme (complex I) (A rotenone-like reaction).

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3. Materials and Methods

3.1 Materials

We bought M8OI (the Cl− salt, > 96% purity) from Sigma (Poole, UK). The following products were acquired from Sigma-Aldrich (Burlington, USA): dimethyl succinate (98% purity), menadione (vitamin K 3), chlorpromazine hydrochloride (CPZ) (≥ 98% purity), rotenone (95% purity), diodium succinate (99% purity), dicoumarol, ketoconazole, succinyl glycine, and dimethyl sulphoxide (DMSO). Menadiol, or vitamin K 4, was bought from BioTek (Pasadena, USA) and apigenin (≥ 98% purity) from Abcam (Waltham, USA).

3.2 Methods

3.2.1 Cell Culture and Treatment

3.2.1.1 Culture of adherent AR24J -B-13 cell line

Rat B-13 pancreatohepatic progenitor cells were cultivated in Dulbecco's modified eagle culture medium (DMEM) from Sigma Aldrich in 24 and 6 well plates at 0.5 and 2 ml, respectively. The media contained 10% (v/v) fetal calf serum (FCS), 80 µg/ml L-glutamine, and 1 gm;L glucose with 80 units/ml of streptomycin and penicillin. Cells were divided every two to three days and cultivated in an automated humidified incubator at 37°C with 5% CO 2 continuously introduced into the media.

3.2.1.2 Cell splitting

Cells were passaged once confluence was reached. The cells were cleaned with sterile 1x phosphate buffer saline (PBS) after the cell medium was removed. After

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that, the cells were treated with diluted trypsin-EDTA in PBS for five minutes, or until they separated from the flask. Sometimes, certain cells were unable to separate; in these cases, the cells were moved with cautious, light strikes. Eight milliliters of the growth medium were added to the trypsin to make it inactive once the cells had fully separated. The cell suspension was centrifuged at 20,000 rpm for five minutes. The cell pellet was resuspended in a new medium and the supernatant was discarded prior to reseeding.

3.2.1.3 Cell counting using trypan blue exclusion

Trypan blue staining, which is incorporated into dead or damaged cell membranes but is rejected by living cells, was used to measure the quantity and vitality of cells. Following removal, the cell culture was carefully washed in PBS (137 mM NaCl, 27 mM KCl, 100 mM phosphate pH 7.4) and then trypsinized (50 mg/L in PBS). The number of living and dead cells was then counted using a hemocytometer by mixing trypan blue solution (sterile-filtered 0.4% (w/v) trypan blue in 137 mM NaCl and 3.4 mM K 2 HPO 4 pH 7.4) in a 1:1 ratio with the cell suspension.

3.2.1.4 Cells treatment

To ensure that the seeded cells adhered to the plate foundation, they were kept in an incubator for a full day. Stock solutions of the compounds were prepared using ethanol, water, or DMSO, depending on the solubility of the compounds. These solutions were either utilized right away or stored at -20°C. Fresh media was utilized to prepare stocks at a ratio of 1:1000. Before adding the newly made solutions containing the therapy to be evaluated, the old media was taken out of

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each well. After then, the experiment dictated how long the plates should stay in the incubator.

3.2.2 Thiazolyl blue tetrazolium bromide (MTT) assay

Following therapy, the old medium was thrown out and replaced with a 1:10 dilution of 5-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in PBS, which was then incubated for two hours. After removing the MTT medium, each well was filled with 0.5 ml of isopropanol, and the cells were gently shaken for 10 minutes on a plate shaker. Before deducting the 690 nm reading from the 570 nm reading, absorbance was measured at 570 and 690 nm.

3.2.3 Protein isolation and quantification

3.2.3.1 Preparation of cell extract

In order to maximize the protein concentration per sample, cells were cultivated in six-well plates with three of the wells treated with the same chemical and combined into a single sample. Prior to removing the media containing the substance to be tested and twice washing the samples with 1 milliliter of sterile PBS, the cells were scraped off of the well wells using 1 milliliter of PBS. After that, the cell suspension was moved to a sterile Eppendorf and centrifuged for five minutes at 13,000 rpm. After removing the supernatant, the cell pellets were once again suspended in the extraction buffer, which contained a protease inhibitor cocktail (1:1000) and 100mM Tris-HCL pH 7.4, 100mM NaCl, 2mM EDTA pH 8.0, 25mM NaF, 0,1% Triton, 1mM Benzamidine, and 0.1mM Na3VO4. After fragmenting the cells with sonication, the contents were centrifuged once more to

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gather the supernatant. After that, the supernatants were kept for further examination at -20 °C.

3.2.3.2 Quantification of protein by lowry assay

Bovine serum albumin (BSA) was used in the Lowry assay to measure the protein content of the sample, and standards ranging from 0 to 20 µg/ml were made. dH 2 O and ABC buffer (A: 2% w/v NaCO/4% w/v NaOH, B: 2% w/v sodium titrate, C: 1% copper sulphate combined in a ratio of 100:1:1) were used to dilute 5µl of the samples and standards for ten minutes. After the incubation period ended, Folin's reagent was diluted one to one in dH 2 O and incubated for 30 minutes at room temperature (RT).

3.2.4 SDS-page and Western blotting

3.2.4.1 SDS-polyacrylamide gel electrophoresis (PAGE)

0.05% v/v TEMED, 0.1% w/v SDS, 0.05% w/v ammonium persulphate, and 375 mM Tris buffer pH 8.8 were combined to create a 9% acrylamide gel. Additionally, 4% acrylamide, 0.1% w/v SDS, 0.05% w/v ammonium persulphate, 0.1% v/v TEMED, and 125mM Tris buffer pH 6.8 were combined to create a stacking gel. After that, gels were put into gel tanks and filled with the electrode running buffer, which included 20 mM Tris, 160 mM Glycine, and 0.08% w/v SDS at pH 8.3. After adding 15µl of a color burst marker and protein sample to each well, the gel was run for 15 minutes at 90 V and subsequently for 50 minutes at 120 V.

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3.2.4.2 Electrotransfer

Next, proteins were transferred from the gel to a membrane made of nitrocellulose. The nitrocellulose and gel were put into the transfer module, which had been previously filled with chilled transfer buffer (final pH of 8.3 ± 0.3) that contained 192 mM glycine, 25 mM Tris, and 20% v/v methanol. In order to remove methanol from the transfer, the electrotransfer was run at 160 V for 60 minutes. After that, the nitrocellulose was rinsed three times for five minutes each in TBS-T, which is made up of 20 mM Tris, pH 7.4, 0.05% v/v Tween 20, and 0.2 M NaCl. This was then blocked for an hour at room temperature using a blocking buffer made of semi-skimmed milk powder (3% w/v milk powder) and TBS-T.

3.2.4.3 Immunodetection

The nitrocellulose membrane was once more rinsed in TBS-T buffer three times apiece for five minutes after blocking. The dilutions of the primary antibody are displayed in Table 1. The incubation buffer, which was made up of 0.3% w/v milk powder and TBS-T, was then added. The mixture was then kept on a rotating shaker at 4 °C. The nitrocellulose was cleaned once more, and after adding the secondary antibody and incubating it for an hour, each wash cycle lasted five minutes.

3.2.5 Seahorse flux studies

Using the Seahorse XF analyser XFe96 (Agilent, Stockport, UK), the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured to study glycolysis and mitochondrial respiration, respectively. Assays using intact cell membranes, 1 mM sodium pyruvate, 5.5 mM glucose, 2 mM glutamate, and

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pH 7.4 adjustment were carried out in Agilent medium. B-13 cells were plated in XFe 96-well plates at a density of 15,000 cells per well, and they were cultivated for an additional night. Following an injection of carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), rotenone/antimycin A, and oligomycin, compounds of interest were incubated for one hour in a 3-14-3 min measurement cycle. Three cycles of 2-3-2 min were then conducted.

3.2.6 Statistics

For statistical analysis, GraphPad Prism (version 9.4.0) was utilized. Unless otherwise indicated, the results are given as mean ± STD n = 3. After doing a one-way analysis of variance (ANOVA), a Tukey Kramer LSF post hoc analysis was used to identify significant differences. Statistics were considered significant when $P < 0.05$.

4. Results

4.1 Disodium succinate showed a significant inhibition of M8OI toxicity by increasing the mitochondrial MTT reduction capacity in a dose-dependent manner in B-13 cell

The MTT tetrazolium reduction assay was used as a quantitative and sensitive way to detect cell viability after exposure to the compounds of interest at different concentrations of M8OI (5 µM and 10 µM) in order to investigate the mechanism(s) of M8OI toxicity and identify compound(s) that antagonize/reduce M8OI toxicity (Youle and Bliek, 2012). Two concentrations of M8OI were employed for all MTT studies, based on the EC 50% for M8OI determined by

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Probert et al. (2018): 5 µM to lower cell viability by about 50% and 10 µM to induce a higher toxicity.

In B-13 cells, all chemicals were studied for 24 or 48 hours at a range of concentrations (0–10 mM), with three repetitions for each study. Compounds of interest were treated with B-13 cells both before to and during exposure to M8OI (5 µM or 10 µM) for one hour. Since CPZ (200 µM) is known to cause cell death quickly, it was utilized as a positive control. With the exception of apigenin at 40 and 80 µM (P-value 0.007, 0.01), none of the tested chemicals reduced the viability of B-13 cells at the utilized doses. This is probably because apigenin is known to induce mitophagy at ≥ 40 µM (Filadi et al., 2018). Disodium succinate at 5 and 10 mM, however, significantly increased the apparent vitality of B-13 cells throughout the course of a 24-hour research as compared to the vehicle control.

When compared to the vehicle control, the viability of B-13 cells was considerably reduced by 5 and 10 µM M8OI to about 60% and 80%, respectively (Figures 3B, C, E, F, and H). But in 24 and 48 hours of exposure, only disodium succinate demonstrated dose-dependent, significant (P-value ˂0.05) protective effect against M8OI toxicity (at 5 and 10 µM) (Figure 3B and C). The toxicity of M8OI (10 µM) was not significantly reduced by the other studied succinate derivatives, succinyl glycine or dimethyl succinate (Figures 3G and H). However, in comparison to 10 µM M8OI, only 1 mM dimethyl succinate demonstrated substantial protective action (P-value 0.02). Nevertheless, no further research was carried out (at lower 5 µM M8OI and during 48 hours exposure) since the chemical ran out in the host laboratory.

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4.2 M8OI targets mitochondrial ETC, probably via causing inhibition of complex I-dependent NADH oxidation

According to our theory, M8OI inhibits mitochondrial ETC, which in turn inhibits complex l, which inhibits NADH oxidation, and an imbalance (higher) $NADH/NAD^+$ ratio, which inhibits oxidative phosphorylation. The DT-diaphorase enzyme, which is present in B-13 cells, is known to be able to oxidize NAD(P)H when substances like menadione are present (Wallace et al., 2010). B-13 cells were treated with varying dosages of M8OI plus menadione or menadiol with/without dicoumarol in order to study this impact. B-13 cell viability was measured using the MTT test.

Thus, a dose-response toxicity in high (4500 mg/L) and low (1000 mg/L) glucose concentrations media was conducted to establish a non-cytotoxic concentration range, to examine these compounds and their potential protective activity against M8OI toxicity in detail. To date, no studies have used B-13 cells to assess the non-cytotoxic concentration of menadione, menadiol, and dicoumarol.

When B-13 cells were exposed to 5 µM M8OI, their MTT assay results were significantly lower than those of the vehicle control. Adding menadione or menadiol did not significantly mitigate the harmful effects of M8OI. On the other hand, a substantial protective activity (P-value˂0.05) of menadione and menadiol at 100 nM concentration was seen in B-13 cells at 10 µM M8OI.

With the exception of dimethyl succinate, which only had one repetition, the data represent the mean of three exposures. It's interesting to note that the addition of 5

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µM dicoumarol counteracted the protective effects of menadione and menadiol at concentrations of 100 nM. Further examination was carried out at 100 nM menadione and menadiol with/without 10 µM M8OI and 5 µM dicoumarol in order to verify their reported protective efficacy against 10 µM M8OI. This was in line with earlier research as 5 µM dicoumarol blocks the effect of 100 nM menadione and menadiol, which dramatically lower 10 µM M8OI toxicity (P-value˂0.05).

4.3 M8OI targets mitochondria ETC and inhibit mitochondrial oxidative phosphorylation in B-13 cell

Our hypothesis proposed that M8OI targeted mitochondrial ETC, causing suppression of oxidative phosphorylation likely via inhibition of complex l. Current investigations by the host laboratory demonstrated that M8OI targeted mitochondria, probably via interaction with ETC. Therefore, utilizing the Seahorse XF analyser XFe96, a more thorough examination of the mitochondrial function was carried out. Different concentrations of M8OI (10 µM, 50 µM, and 100 µM) were used to investigate the effects of M8OI on mitochondria in intact B-13 cells.

OCR was inhibited at 10 µM M8OI when complex V activity was blocked by oligomycin, but at 50 and 100 µM M8OI, there were no changes since the OCR was already completely inhibited. Uncoupler (FCCP) addition resulted in an OCR that was marginally greater than that of a typical vehicle. However, at 50 and 100 µM M8OI, ATP generation and proton leak (related to OCR) were totally suppressed, although at 10 µM, these effects were around 50% lower than in the normal control.

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4.4 Disodium succinate improved cellular respiration even at highest used M8OI concentration (100 µM)

Using the Seahorse XF analyzer, mitochondrial function study was done to look into the protective effect of disodium succinate against M8OI poisoning. Disodium succinate's protective ability was investigated at 10 mM since it demonstrated the best defense against M8OI toxicity. Furthermore, the experiment was carried out at 10, 50, and 100 µM M8OI, at circumstances akin to those of earlier seahorse analyses.

10 mM disodium succinate considerably reduced the M8OI inhibitory effect on OCR in B-13 cells at 10, 50, and 100 µM M8OI doses (P-value 0.05). Furthermore, at three distinct doses, 10 mM disodium succinate considerably decreased the toxicity of M8OI on ATP generation and proton leak (related to OCR) (P-value 0.05). However, because the standard error in the treated groups was somewhat high (around 20), no significance was seen.

4.5 M8OI induction of apoptosis were mediated by mitochondrial pathway

According to published research, M8OI causes apoptosis in a range of cell lines. Western blot examination of pro-apoptotic protein (cytochrome c) expression in the cytosolic and mitochondrial fractions of B-13 cell was done to determine whether M8OI-mediated mitochondrial pathway causes apoptosis. Dr. Tarek Abdelghany contributed the fractions for the analysis. Blotting of B-13 cell cytosolic and mitochondrial fractions after 6 hours of treatment with varying concentrations of M8OI, 10 µM rotenone, and 1 µM staurosporine was done to test

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our hypothesis. As positive controls for apoptosis induction, staurosporine and rotenone were used (Li et al., 2003; Belmokhtar et al., 2001).

4.6 M8OI probably induce mitophagy in B-13 cells via PINK1-Parkin signalling pathway

Previous research suggests that M8OI causes disruption of mitochondrial activity in different cell lines, which in turn causes intracellular metabolic imbalances and ultimately cell death. In the past, we postulated that the induction of mitophagy as a cytoprotective cellular mechanism against M8OI-induced mitochondrial breakdown may be one of the effects of the compound.

In order to verify our theory, B-13 cells were subjected to varying concentrations of M8OI, 20 µM ketoconazole, and 2 µM thapsigargin. Following the protocol, protein samples were taken at 3, 6, 12, and 18 hours of treatment (Section 6.2.3.1). Since ketoconazole is known to induce mitophagy (Chen et al., 2019), it was utilized as a positive control; in contrast, thapsigargin is known to induce mitochondrial dysfunction (Zhang and Ren, 2011). Consequently, protein expression studies of PINK1, parkin, and MFN2 were carried out using Western blot techniques.

In comparison to B-13 cells that were not treated (control), the data show a relative increase in PINK1 and MFN2 expression in B-13 cells treated with the various concentrations of M8OI and the aforementioned chemicals. Furthermore, at 1 and 10 µM M8OI, Parkin expression was slightly elevated after 3, 6, and 12 hours of treatment. Thus, a quantification of protein expression using the ImageJ browser's

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β-actin normalization is necessary to validate the difference in protein expression. But Parkin's look was ambiguous, so it's impossible to measure.

5. Discussion

5.1 Discussion of results

5.1.1 Reduction of M8OI toxicity

Thus far, no published study has shown any chemicals that can either fully or partially detoxify M80I. Although succinate derivatives have been studied as possible agents with potential detoxifying properties, none of them have demonstrated a substantial ability to lower M8OI toxicity to yet (Abdelghany et al., manuscript in process). On the other hand, disodium succinate was discovered in this experiment for the first time as an inhibitor of M8OI's harmful effects in B-13 cells. The findings consequently corroborate our theory that M80I inhibits oxidative phosphorylation by interacting with the mitochondrial extracellular trapping complex 1, most likely through this mechanism.

The comprehension of succinate's function in oxidative metabolism leads to these deductions. When succinate interacts with the mitochondrial ETC, it functions as a tricarboxylic acid (TCA) intermediate and "shortcuts" the generation of ATP through oxidative metabolism (Giorgi-Coll etal., 2017). Complex II (succinate dehydrogenase (SDH)) converts succinate to fumarate during the TCA cycle. The comprehension of this occurs on the inner mitochondrial membrane (Bakare et al., 2021), and it serves as the foundation for the outcomes. Additionally, Giorgi Coll

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et al. (2017) observed a 20% increase in OCR when succinate is given to mixed glial cells in the presence of the recognized complex 1 inhibitor rotenone.

Furthermore, Piel et al.'s (2020) in vitro research employing human hepatocyte cells demonstrated that prodrug (NV241, cell permeable succinate) prevented mitochondrial complex I-related dysfunction and promoted oxidative phosphorylation, thereby saving coupled respiration through the transfer of electrons from complex II to complex III. These results are consistent with the research presented in this work since it was demonstrated that 10 mM disodium succinate increased OCR and parameters related to OCR, such as proton leakage and ATP generation, and decreased ECAR in B-13 cells when exposed to different doses of M8OI.

5.1.2 M8OI probably inhibit complex l-dependent NADH oxidation

Menadione (vitamin K3), a naturally occurring chemical molecule, is converted to menadiol (vitamin K4) in hepatocytes by NAD(P)H:ubiquinoneoxidoreductase (DT-diaphorase), which uses both NADPH and NADH as electron donors (Livingstone et al., 1989; Yamashoji., 2016). This study proposed that M8OI inhibits the oxidative action of NADH via complex I, which results in an imbalance in the NAD(P)H/NAD(P)+ ratio and suppression of the ETC. Because this would inevitably result in a higher level of oxidative stress and oxidative damage, mitochondria cannot function properly if appropriate ratios are not maintained (Wu et al., 2016; Stein and Imai, 2012).

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Menadione may be helpful in complex I deficiency, according to the evidence (Wijburg et al., 1990). Furthermore, it has been demonstrated by (Dedukhova and Mokhova, 1987) that menadione enhances ATP generation in vitro, which in turn raises cellular respiration when rotenone is present. The results of our study were in line with other research as menadione at 100 nM dramatically decreased the toxicity of 10µM M8OI. It's interesting to note that menadione's detoxifying ability was hindered when dicoumarol, a DT-diaphorase blocker, was added. This shows menadione works by preserving an ideal NAD(P)H/NAD(P)$^+$ ratio. Thus, since NADPH consumption is restricted to DT-diaphorase activity, which is the conversion of menadione to menadiol, we can infer, based on the work of Livingstone et al. (1989).

Furthermore, at 10 µM M8OI, 100 nM of menadione was demonstrated to have significant protective properties ($P < 0.05$), while there was no discernible decrease in toxicity at half the dose of M8OI, or 5 µM. As a result, the ratio of NAD(P)/NAD(P)$^+$ is unaffected by M8OI at lower doses. Although rotenone appears to block the oxidoreductase activity of complex I NADH, its route of toxicity has not been conclusively demonstrated (King et al., 2009).

5.1.3 M8OI induces apoptosis probably via mitochondrial pathway

The two pathways that lead to apoptosis are the intrinsic "mitochondrial" pathway and the extrinsic "death receptor" pathway. The mitochondrial pathway is triggered by various stressors such as toxins, radiation, free radicals, and metabolic stress, while the extrinsic pathway is triggered by transmembrane death-receptors (such as the tumour necrosis factor (TNF) receptor family) (Elmore, 2007).

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The agents that trigger the mitochondrial-apoptotic pathway generally cause changes to the inner mitochondrial membrane, which lead to the release of two classes of pro-apoptotic proteins that are sequestered within the mitochondria, the loss of transmembrane mitochondrial potential, and the opening of the mitochondrial permeability transition pore (MPTP) (Elmore, 2007). The caspase-dependent apoptotic pathway is responsible for the first category of these proteins to be released, which includes the translocation of cytochrome c from mitochondria to the cytosol.

Apoptosis-inducing factor (AIF) and endonuclease G comprise the second, which is a part of the caspase-independent apoptotic pathway (Bröker et al., 2005). The latter are released when irreversible cell death occurs (Elmore, 2007). Thus, a crucial signal for the initiation of mitochondrial apoptosis is the release of cytochrome c into the cytosol (Gogvadze et al., 2006).

5.1.4 M8OI toxicity is probably involving induction of mitophagy as a protective cellular mechanism

The primary source of eukaryotic adenosine triphosphate (ATP) synthesis, mitochondria function as the cell's energy factory (Murphy, 2009). Stress-induced mitochondrial damage can trigger apoptosis by a process known as mitochondrial outer membrane permeabilization, or MOMP (Osellame et al., 2012). To lessen the potentially lethal effects of this, cells use mitophagy to clean depolarized mitochondria before inducing MOMP (Ma et al., 2020). The two main components of this mitophagy-related mitochondrial regulatory signaling pathway are Parkin and PINK1 (Ge et al., 2020). Phosphorylation of apoptotic regulatory proteins,

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most notably BCL2, is elevated when the mitophagy mitigatory mechanism is no longer viable, activating controlled cell death (Wei et al., 2008).

To the best of our knowledge, this is the first study looking into the induction of mitophagy by MILs. The results indicated that MFN2 and PINK1 were significantly overexpressed following exposure to various concentrations of M8OI at different times compared with the vehicle control (P-value <0.05), suggesting that the mechanism of M8OI toxicity involves induction of mitophagy as a cytoprotective mechanism via activation of the PINK1-Parkin pathway.

It has been demonstrated that when mitophagy is activated, protein concentrations associated with it, particularly those of Parkin, MFNs, and PINK1, rise (Yapryntseva et al., 2022). Parkin expression was not immediately apparent in our investigation, although this can at least be partially explained because the exact mechanisms behind Parkin are yet unknown (Ma et al., 2020). However, it is known that Parkin translocates from the cytosol to depolarized mitochondria upon activation (Ma et al., 2020). According to Yapryntseva et al. (2022), studies examining parkin expression in cell fractions yield more precise results. Consequently, greater research on parkin expression in mitochondrial fractions will aid in elucidating the function of mitophagy in M8OI-dependent cell death.

The PINK1/Parkin pathway has been compared to the kinetics of mitochondrial fission and fusion in other research (Kubli and Gustafsson, 2012). Moreover, it has been demonstrated that the mitochondrial fusion proteins Mfn1 and Mfn2 target and are crucial in Parkin-mediated ubiquitination once mitophagy is induced

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(Basso et al., 2018). This explains the study's findings since Parkin assisted in the proteasomal breakdown of the mitochondrial outer membrane protein (Mfn2) when Mfn2 was overexpressed.

5.1.5 Inhibitory effect of M8OI on oxidative phosphorylation

OCR and ECAR can be used to measure the two main processes of cellular ATP production: glycolysis and mitochondrial oxidative phosphorylation (OXPHOS). Our results indicated that the concentration of M8OI affected the suppression of mitochondrial OXPHOS. In other words, OCR was lowered by about 80% at 50 and 100 µM M8OI compared to 50% at 10 µM M8OI. The first events following M8OI exposure that have been seen are the suppression of mitochondrial OXPHOS, and it is most likely the interaction between M8OI and the mitochondrial ETC that serves as the main beginning effect in the toxicity of M8OI. When OXPHOS is inhibited, ATP generation is lost, which probably led to an increase in compensatory glycolysis as seen by an increase in ECAR.

Since oxygen consumption in B-13 cells is almost 50% reduced after adding a lower concentration of M8OI (10µM), it is likely that the lower doses of M8OI targeted mitochondrial ETC, interrupting electron transfer without inhibiting OXPHOS (NADH oxidation). On the other hand, adding FCCP in the presence of oligomycin (to block proton passage via complex V) results in uncoupled oxygen consumption, indicating similar levels of proton pumping despite inhibited oxygen consumption (interruption of electron flow between complex I and complex III).

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Given that at 50 and 100 µM M8OI, OCR and OCR-related parameters (ATP synthesis and proton leak) were totally blocked. Both intrinsic and extrinsic mechanisms may eventually play a role in mediating apoptotic cell death. In this regard, ROS formation at higher and hazardous concentrations of M8OI in different cell lines has been documented by other researchers (Ma et al., 2018; Li et al., 2015; Jing et al., 2013). Furthermore, a greater NADH/NAD^+ ratio is thought to be one of the primary causes of a significant amount of mitochondrial H 2 O 2 outflow (Murphy, 2009).

5.1.6 Toxicological importance

Because PBC is a complicated disorder, doubts about its aetiology persist despite substantial knowledge of the genetic and environmental components linked to vulnerability to the condition. To make matters even more complicated, there isn't much information available in the literature about M8OI because it's not used in cosmetic, household, pharmaceutical, or pesticide goods. As a result, M8OI's uses are restricted to industrial processes and applications. Alternatively, it may be used as an undeclared component of goods that aren't typically meant to come into contact with humans, such battery additives.

However, after a research of 20 PBC patients and 10 controls, greater concentrations of soil M8OI have been associated with a higher prevalence of PBC (Probert et al., 2018; Leitch et al., 2021). This study shows more proof in favor of earlier research on the subject, showing that M8OI is toxic to liver progenitor cells due to its capacity to cause mitophagy and apoptotic mood of cell death by

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interfering with ETC in mitochondria, which in turn disrupts oxidative phosphorylation.

6. Limitations of methodology and future work

A few constraining elements related to the current project's methodological approaches could negatively affect the applicability of the suggested conclusions. First off, the most obvious restriction is that M8OI toxicity in hepatic cells was studied using a hepatic progenitor model (B-13 cell) rather than primary hepatocytes. While there are several benefits to using B-13, including as its affordability and ease of culture (Probert et al., 2015), it is difficult to presume that the intracellular activities taking place in these cells are a true representation of those occurring in primary hepatocytes. Furthermore, compared to primary hepatocytes, B-13 cells have fewer mitochondria (Probert et al., 2014). Consequently, these limitations would be addressed by repeating this investigation with primary human hepatocytes or B-13H cells.

Second, no time variations in protein expression upon M8OI administration were investigated since cytochrome c expression was only evaluated once, at the end of a 6-hour period. A more definitive set of results could be obtained by employing western blotting to measure cytochrome c expression at different time intervals. Finally, a more comprehensive understanding of the involvement of the PINK1-Parkin pathway in the induction of mitophagy would be obtained by analyzing the expression of PINK1 and Parkin in isolated mitochondrial fractions of B-13, B-13/H, or primary hepatocyte cells upon exposure to M8OI.

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As mentioned earlier, research indicates that long-term exposure to xenobiotics could be a substantial risk factor for PBC. Thus, in addition to the previously mentioned additional works, we strongly advise carrying out long-term in vivo studies that span two years to examine the effects of non-lethal concentrations of M8OI on rodents. These studies are likely to yield a more conclusive set of results that may eventually be extrapolated to effects on human health. It would be ideal for this study to track changes in blood immunological levels and liver phenotyping across certain time periods that are connected to PBC.

7. Conclusion

According to the evidence in this research, complex I of the mitochondrial ETC processes in B-13 cells is the target of M8OI, which inhibits mitochondrial oxygen consumption via two different mechanisms. These consequences are most likely the main starting points for the cytoprotective process of mitophagy activation and the final induction of apoptosis. Significantly, our study demonstrated that disodium succinate decreased ECAR and increased OCR and OCR-associated parameters (ATP generation and proton leak) in a dose-dependent manner, thereby mitigating M8OI toxicity. However, monitoring the downstream reactions to M8OI and structurally related compounds could help to clarify how xenobiotics drive PBC to PBC over certain time intervals.

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